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Neonatal Neutrophil Inflammatory Responses: Parallel Studies of Light Scattering, Cell Polarization, Chemotaxis, Superoxide Release, and Bactericidal Activity

B. Wolach,* D. Sonnenschein, R. Gavrieli, O. Chomsky, A. Pomeranz, and I. Yuli

Department of Pediatrics, The Pediatric Hematology Unit and The Laboratory for Leukocyte Functions, Meir General Hospital, Sapir Medical Center, Kfar Saba, Israel and Sackler School of Medicine, Tel Aviv University, Tel Aviv, Israel

Neutrophil dysfunction among newborn infants, especially those born prematurely, is well recognized, but the mechanism responsible for this phenomenon is yet to be clarified. In this study, we evaluated the stimulus response coupling in neutrophils from 90 healthy newborns and 96 healthy adults in an effort to establish whether defective neonatal neutrophil function is a result of impaired signal perception or immature responsiveness. Measurement of rapid- and slow-light scattering responses (LSR) to 1 μM FMLP stimulation revealed that neonatal neutrophils have about one-half the corresponding responsiveness of adult cells (rapid-LSR: 6.1 ± 3.1 arbitrary light intensity units vs. 12.0 ± 2.8, *P* < .001; and slow-LSR: 5.0 ± 2.5 vs. 9.1 ± 2.0; *P* < .001). The same markedly reduced activity was observed in newborn neutrophil chemotaxis and bactericidal activity in comparison with adult cells. Nevertheless, low FMLP concentrations (less than 1 nM) induced no difference in cell polarization between newborn and adult neutrophils, yet at higher FMLP concentrations, the newborn revealed significantly reduced cell polarization. Our data suggest that newborn infants bear a fully functional FMLP signal perception but lack the full capacity of inflammatory responsiveness. Am. J. Hematol. 58:8–15, 1998.

Key words: bactericidal activity; cell polarization; light scattering responses; neonatal neutrophil chemotaxis; neutrophil dysfunction

INTRODUCTION

Newborn infants, particularly those born prematurely, are more likely to develop severe pyogenic infections than older infants or adults [1,2]. This predilection probably results from humoral, phagocytic, and/or cellular immunological deficiencies in the neonate [3,4]. Leukocyte dysfunction [5–7] including poor chemotaxis [8–10], defective adherence and aggregation [11–13], and insufficient opsonization have been reported to occur in the neonatal period [3,4,14–16]. The causes for the leukocyte defects have not yet been fully resolved, but may reflect, among other causes, an incompetent cellular apparatus [17,18] or inadequate signal perception.

The density of the receptors for N-formyl-L-methionyl-Leucyl-L-phenylalanine (FMLP) on the neonatal neutrophil surface has been reported to be comparable to that found on adult neutrophils [19]. However, membrane fluidity, which is known to regulate signal perception [20,21], has been found to be increased in neonatal neutrophils [22], and as a consequence leads to blunted neutrophil responses.

Measurement of light scattering [23], which includes reading both the incident and perpendicular responses, has proven itself to be unique in that it is capable of assessing the very early neutrophil response to chemoattractant stimulation, since it monitors the initial membrane and cytoskeleton rearrangements following cell stimulation. Furthermore, this assay has the advantage of

*Correspondence to: B. Wolach, Department of Pediatrics, Meir General Hospital, 44281 Kfar Saba, Israel.

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distinguishing between inflammatory signals that are perceived by the single FMLP receptor, and are differentially directed to either chemotaxis or bactericidal response pathways. The latter quality enables us to establish whether the response inhibition is due to impaired cell machinery mechanisms (e.g., motility, secretory or oxygen burst mechanisms), or to blunted receptor performance. As far as we know, the perpendicular LSR has not yet been applied to the study of neonatal neutrophil responses. Hence, by using this particular LSR methodology, we expected to ascertain whether the deficient inflammatory responses of neonatal neutrophils could be attributed to the receptor signal perception or to the downstream cellular response-apparatus.

PATIENTS AND METHODS

Subjects

Ninety newborn infants were included in various stages of this study. All were full-term infants born vaginally to healthy mothers after normal pregnancy and delivery. Their birth weight ranged between 2,800–3,400 g, and their Apgar scores were 9 and 10 at 1 and 5 min, respectively. Blood samples were taken at 2–5 days after birth. The study was approved by the Helsinki Committee at the Meir General Hospital, Sapir Medical Center, Kfar Saba, Israel. A control adult study group consisted of 96 healthy volunteers aged 20–45 years.

Reagents

N-formyl-L-methionyl-L-leucyl-L-phenylalanine (FMLP), Phorbol myristate acetate (PMA), N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid (HEPES), superoxide dismutase, ferricytochrome-C, bovine serum albumin, Krebs-Ringer phosphate buffer (KRP), and phosphate buffer saline (PBS) were purchased from Sigma Chemical Co. (St. Louis, MO). Earle's salt solution supplement with L-glutamine (M199 medium) was purchased from Biological Industries Inc. (Kibbutz Beit Haemek, Israel). Hank's Balanced Salt Solution (HBSS) was prepared by the Weizmann Institute Biological Services. Dextran T-250 was purchased from Pharmacia Fine Chemicals (Pharmacia Laboratories, Pitscataway, NJ). Heparin (5,000 U/ml) was purchased from LEO Ltd. (Ballerup, Denmark).

Polymorphonuclear Leukocyte (Neutrophil) Isolation

Five milliliters of heparinized peripheral blood from newborns, or 20 ml from adults, was mixed with equivalent volumes of 3% dextran (T-250) in saline. Neutrophils were isolated according to Boyum [24], and residual erythrocytes were removed by hypotonic lysis. The neutrophils were then suspended in HBSS supplemented with 10 mM HEPES (HHB), pH 7.4, for light scattering assays, in M199 medium for chemotaxis assays, in Krebs-Ringer buffer (KRP) for superoxide anion release assays, and in PBS supplemented with 0.2% D-glucose and 1% bovine serum albumin (PBS-GA) for bactericidal activity assays.

Morphological Response

Neutrophil suspensions at 4×10^6 cells in 0.4 ml HHB were vigorously stirred (500 rpm) at 37°C. The neutrophils were stimulated by adding 4 μl of FMLP-solution to achieve final concentrations of 10 pM, 100 pM, 1 nM, 10 nM, 100 nM, and 1 μM. Kinetic studies revealed that the cell polarization is fully apparent 30 sec after stimulation. Cell morphology was captured by the addition of 0.4 ml of ice-cold 10% formaldehyde in HHB, and classified by light microscopy as to whether there were spherical or polar-shaped cells. The percentage of polarized cells in triplicate samples of 500 cells each was determined.

Light Scattering Responses

Incident and perpendicular light scattering responses of neutrophils were measured in a dual aggregation meter (DP-247E; Sienco Inc., Morrison, CO), as previously described [23,25]. Briefly, neutrophil samples of 0.4 ml at 10^7 cells/ml HHB were suspended in 1-ml cuvettes at 37°C. Baseline levels of light scattering from neutrophil suspensions were stabilized prior to stimulation by fine adjustment of the cell stirring in the range of 500 ± 50 rpm. Stimulation was achieved by injecting 4 μL of either 10 nM or 1 μM FMLP. Figure 1 depicts typical patterns of the incident and perpendicular responses. The response of the incident light (lower tracing) is characterized primarily by one relatively slow peak, on the order of 5 min. This effect is quantified by measuring its peak amplitude or the area under the tracing integrated from the time of stimulation to 3 min (integration performed with the aid of the Auto Cad package). The response, as detected by perpendicular scattering (upper tracing), is comprised of two transient peaks. The first, the rapid-light scattering response (rapid LSR), is defined by the 10 ± 1 sec required from stimulation to peak reduction in light intensity, and the additional 10 sec for the restoration of light intensity [23]. The ensuring slow-light scattering response (slow-LSR) yields a peak after about 50 ± 10 sec from stimulation, followed by a rather slow return to pre-stimulated light scattering level, in the order of several minutes. The area above the perpendicular light scattering was integrated for 3 min after stimulation. The possibility of mixing artefacts was negated at the introduction of the light-scattering method [23,25]. This was proven by using cell-free systems, and non-stimulants (buffers). In addition, responses to stimulation with a spectrum of six orders of magnitude of several

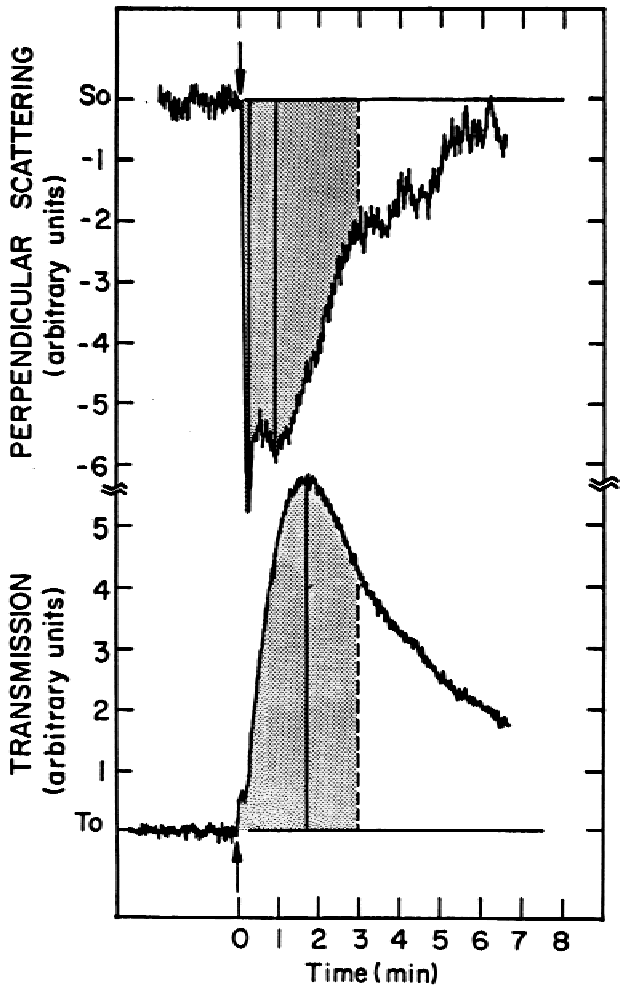

Fig. 1. Light scattering response of a neutrophil suspension to a single stimulation with 10 nM FMLP, which was achieved by an injection at the zero time point (indicated by the arrows). The response is characterized by a single incident light wave (peaks at approximately 1 min; bottom), and two sequential low peaks (10 and 40 sec, respectively) of perpendicular scattered light (top). The amplitudes of the responses are depicted by the vertical bars, and the integrations of either the incident or the perpendicular responses of the first 3 min are indicated by the respective shaded areas.

chemoattractants yielded the exact 10 sec peak in every response.

Chemotaxis Assay

A 48-well chemotactic microchamber (Neuro Probe, Inc. Bethesda, MD) was used to determine random migration and chemotaxis [26]. M199 medium or FMLP at a concentration of 10 μM was added to the wells of the bottom plate. A polycarbonate filter sheet, PVP-Free, containing 3-μm pores (Nucleopore Corp., Pleasanton, CA) was placed on top of the bottom plate. A matched upper plate was attached to the bottom one and fixed firmly. Fifty microliters containing 5×10^4 neutrophils (i.e., 10^6 cells/ml) was placed in upper wells. The chamber was incubated for 60 min in a humidified atmosphere at 37°C. After incubation, the cells from the upper side of the filter were wiped off, and the filter sheet was stained with May-Grumwald-Giemsa. The number of migrating cells was determined in nine fields by light microscopy (×40 objective and a ×10 ocular equipped with a fine grid). Net chemotaxis was calculated by subtracting the random migration (M199 medium in the bottom wells) from the FMLP-driven neutrophils. Experiments were carried out in duplicate.

Bactericidal Activity

The maximal bactericidal activity was evaluated as previously reported [27]. Bacteria (*Escherichia coli*) were freshly harvested prior to each experiment, and allowed to enter an early stationary growth phase (18 hr at 37°C). The final density of the bacteria was assessed by spectrophotometry at 590 nm. A suspension of neutrophils at 5×10^6 cells/mL PBS-GA (final density) was incubated with bacteria at a neutrophil bacteria density ratio of 1:5 or 1:10, respectively, in the presence of 10% autologous serum. The incubating systems were agitated gently for 30 or 90 min in a 37°C chamber. After incubation, the neutrophils were lysed with 4 volumes of distilled water, plated (30 mL) in triplicate in broth agar plates, and incubated for 24 hr at 37°C. Each experiment included two controls that were comprised of PBS-GA and bacteria or autologous serum and bacteria. In these experiments, we observed less than 0.02 log decrease in bactericidal activity ($n > 100$) in the control chambers.

Superoxide Anion Release

The assay was carried out as previously reported [28]. Neutrophils at a final density of 5×10^5 cells/mL KRP were preincubated with or without 214 U/mL superoxide dismutase for 5 min at 37°C. Then, 60 μM ferricytochrome-C was added, and the response was initiated by the prompt addition of 0.1 μM FMLP or 1 μg/mL phorbol myristate acetate. The reaction mixtures were incubated for 10 min at 37°C. The reactions were stopped by placing the tubes in melting ice. The neutrophils were centrifuged (500*g* for 10 min at 4°C), and the optical density of the supernatant was determined at 550 nm. The results of triplicate determinations were averaged, and superoxide anion release was calculated according to Massey, using the extintion coefficient of 21,000 M^{-1} cm^{-1}.

Statistical Analysis

Both the Student's *t*-test and the Mann-Whitney nonparametric test were used for data analysis of all leukocyte functions. In addition, the Pearson correlation coef-

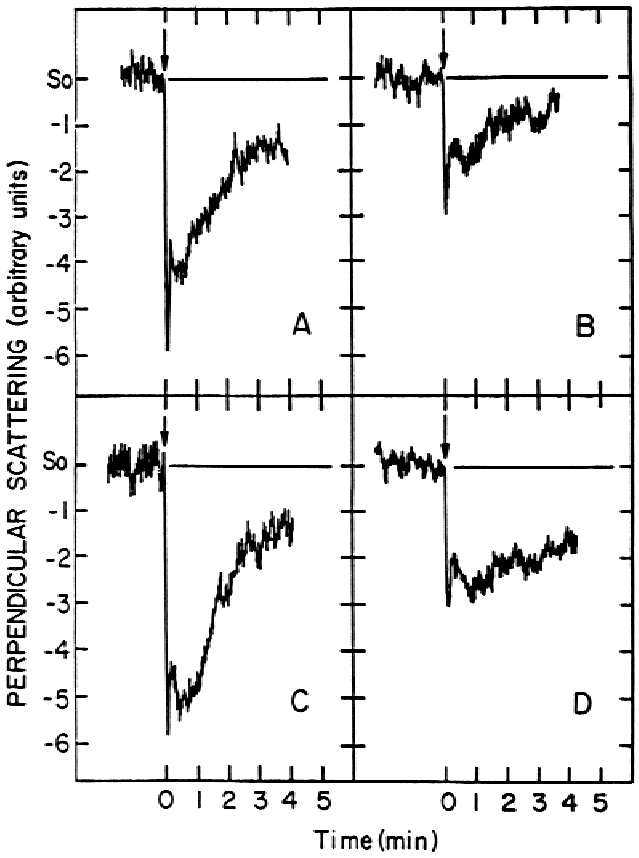

Fig. 2. The perpendicular light scattering responses to 10 nM FMLP (A,B) and 1 μM FMLP (C,D) of adult neutrophil suspensions (A,C) and newborn infant cell suspensions (B,D).

ficient was used for assessing statistical correlations of light scattering, chemotaxis, and bactericidal activity.

RESULTS

The response of neonatal neutrophils to FMLP-stimulation was found to be identical to that of the adult neutrophils with respect to timing and amplitude-ratio (i.e., rapid-LSR/slow-LSR, see Fig. 2). The peak timing of the rapid-LSR and the slow-LSR of both neonate and adult neutrophils was strictly comparable: at any FMLP stimulation dose, the rapid-LSR reached its peak effect at 10.5 ± 0.9 sec (n = 22 newborn neutrophils), and at 10.7 ± 1.0 sec (n = 9 adult cells) after stimulation, while the slow-light scattering responses reached their peaks at 61 ± 15 and 59 ± 10.5 sec, respectively. Stimulation by 10 nM FMLP, the optimal dose for chemotaxis induction, yielded rapid- to slow-LSR amplitude ratios of 1.7 ± 0.5 and 1.9 ± 0.5 for newborn infant and adult neutrophils, respectively. Also, stimulation with 1 μM FMLP, the optimal dose for induction of bactericidal activities, yielded the same rapid- to slow-LSR amplitude ratio, i.e., 1.3 ± 0.2, for newborn and adult cells. These findings indicate that, when compared with adult cells, newborn neutrophils respond properly to FMLP stimulation, as determined by the LSR method [23].

Quantification of neutrophil responsiveness to FMLP stimulation revealed differences between neonates and adults. The amplitude of and the area under the light-output traces (see Fig. 2 and Table I) of neonatal neutrophils were about one half that of the adult cells for both the rapid and slow LSR. These differences reached high statistical significance in the rapid-LSR after stimulation by the either 10 nM or 1 μM FMLP. The same high statistical significance was observed in the slow-LSR at the optimal bactericidal activity dose of 1 μM FMLP. In contrast, there were no differences between adult and neonatal neutrophils in the incident light-scattering response, either in amplitude or in the area under the response curve.

To relate the light scattering results to standard cellular and biochemical assays, we tested neutrophil polarization, chemotaxis and bactericidal activities. Figure 3 depicts the FMLP dose-dependent polarization of adult and newborn neutrophils. As previously observed [29], neutrophil polarization reached its peak at 10 nM FMLP, with some decline in the percentage of polarized cells at higher doses. We observed that polarization of adult and newborn neutrophils was practically indistinguishable at concentrations of FMLP lower than 1 nM. At higher concentrations, however, newborn neutrophils revealed significantly less cell polarization.

Chemotaxis was found to generally follow the observations in the cell polarization experiments: chemotaxis of newborn neutrophils was only one half that of adult cells (Table II). However, since the random migration of both adult and neonatal cells was almost identical, net chemotaxis of the newborn neutrophils was only one-third of that of adult cells (see Table II). Similarly, the bactericidal activity of the newborn neutrophils reached one-third the bactericidal capacity of adult cells (Table III, at 90 min).

Significant statistical correlation was found between the rapid-LSR and neutrophil chemotaxis ($P < 0.01$), and slow-LSR and bactericidal activity ($P < 0.01$).

Nevertheless, as previously reported [19], we also observed that superoxide anion generation in response to FMLP (e.g., 0.1 μM) of neonates exceeded that of the adult cells, i.e., 1.9 ± 0.7 (n = 10) and 1.4 ± 0.5 (n = 14) nmol/min/10^6 cells, respectively ($P < 0.005$). However, the 1 μg/mL PMA-stimulated superoxide production in adult and newborn cells was similar (i.e., 6.0 ± 2 (n = 15) and 7.0 ± 1.4 (n = 24) nmol/min/10^6 cells, respectively).

TABLE I. Comparison of Neonatal (n = 22) and Adult (n = 9) Neutrophils' Light Scattering Response to 10 nM and 1 μM FMLP*

a.

	10 nM FMLP			1 μM FMLP		
Measure (amplitude)	Newborn infants	Adults	*P* value	Newborn infants	Adults	*P* value
Rapid-LSR	5.6 ± 2.7	9.6 ± 2.2	<.001	6.1 ± 3.1	12.0 ± 2.8	<.001
Slow-LSR	2.7 ± 2.5	5.2 ± 1.7	<.02	5.0 ± 2.5	9.1 ± 2.0	<.001
Incident-LT	3.5 ± 1.9	3.6 ± 1.9	NS	9.6 ± 3.1	10.1 ± 4.7	NS

b.

	10 nM FMLP			1 μM FMLP		
Measure (area)	Newborn infants	Adults	*P* value	Newborn infants	Adults	*P* value
Slow-LSR	7.6 ± 3.1	12.1 ± 3.5	<.005	11.4 ± 5.3	20.4 ± 6.3	<.001
Incident-LSR	7.5 ± 4.2	7.4 ± 3.6	NS	21.5 ± 6.7	23.3 ± 6.9	NS

*Amplitude of response (cm) shown in **a**; area under the curve (cm^2) between stimulation and after 3 min shown in **b** (mean ± SD).

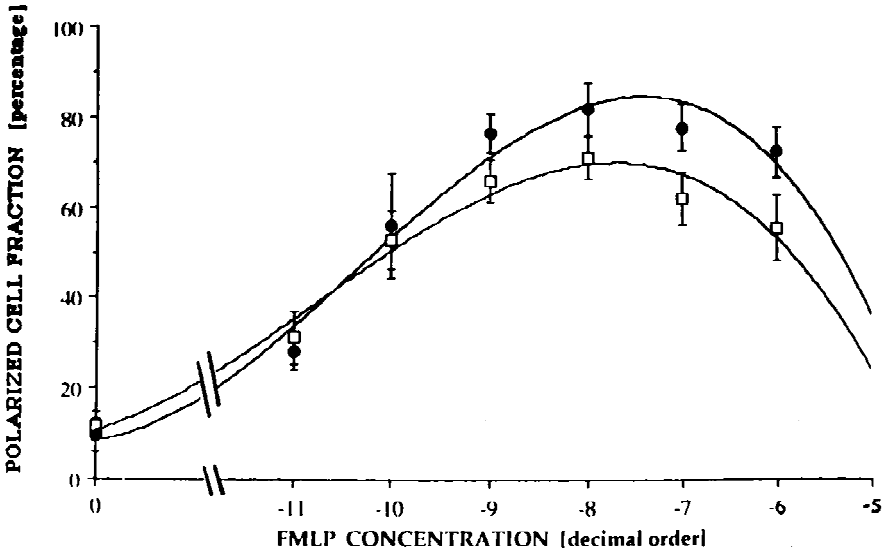

Fig. 3. Whole cell polarization of adult (●) and newborn (□) neutrophils in response to the indicated FMLP concentrations. The experimental points represent the mean and the standard deviation of triplicate samples, each of 500 cells, derived from 10 adult and 10 newborn infant donors. The difference between the adult and the newborn cell polarization reached high statistical significance at FMLP stimulation by 10 nM and higher (*P* < 0.001).

TABLE II. Comparison Between the Neutrophil Migration of Newborn Infants (n = 30) and Adult (n = 40) (Mean ± SD)

Measure	Newborn infants (no. of cells)	Adults (no. of cells)	*P* value
Chemotaxis migration	53 ± 18	108 ± 19	<.001
Random migration	30 ± 14	34 ± 14	NS
Net chemotaxis	25 ± 11	70 ± 14	<.001

DISCUSSION

Neutrophils exhibit a unique behavioral pattern upon stimulation that is manifested in the rapid modulation of their light scattering properties. The perpendicular LSR, in particular, might even distinguish between the early chemotaxis and late inflammatory responses [20,21]. Hence the short and transient characteristic of the rapid-LSR qualifies it as an indicator of chemoattractant signal perception, whereas the slow-LSR indicates degranulation and bactericidal activity.

Although there is still little experimental data to explain the phenomena, we speculate that the light effects are the result of an extremely synchronous and massive rearrangement of organelles in the responding neutrophils.

The light scattering effects are probably a result of two phenomena. The first being the enhanced turnover of microfilaments with the close bundling of F-actin, which then appear as dark spots at the base of the pseudopodia of polarizing neutrophils. This response by microfilaments is one of the earliest and most fundamental neu-

TABLE III. Comparison Between the Neutrophil Bactericidal Activity of Newborn Infants (n = 23) and Adults (n = 23) (Mean ± SD)

	Incubation 30 min (decrease of colonies)			Incubation 90 min (decrease of colonies)		
Cell ratio[a]	Newborn infants	Adults	*P* value	Newborn infants	Adults	*P* value
1:5	0.45 ± 0.10	1.9 ± 0.6	<.001	0.75 ± 0.33	2.4 ± 0.6	<.001
1:10	0.53 ± 0.12	1.7 ± 0.5	<.001	0.70 ± 0.26	2.2 ± 0.6	<.001

[a]Cell ratio = neutrophils: *E. coli*. Decrease in no. of colonies is derived by the following formula: Log (number of *E. coli* colonies without neutrophils/ number *E. coli* colonies with neutrophils).

trophil reactions to surface stimuli [30–34]. The second phenomenon is the discharging of secreting granules of their protein-rich content [35,36].

The observed two-phase, timed response led us to speculate that the rapid-LSR depicts the association of the F-actin fibers into matrix like structures. Actin assembly is generally mediated through G proteins [37,38]. In addition, we speculate that the slow-LSR depicts the secretion event, whereby the secretion involves mechanical movement of all organelles to the plasma membrane and their fusion. Since the secretion organelles are dispersed in the entire cytoplasm, the probability that they would reach the plasma membrane simultaneously is limited. It is, therefore, likely to be a slower and less synchronous event than the bundling of the F-actin fibers.

In this study, neutrophil functions, which are known to be impaired in the neonatal period [3–5,7–13,16], were analyzed to gain a better understanding of the specific neonatal phagocytic impairment at the intracellular level. In addition, the state of the inflammatory response mechanisms themselves was assessed by directly monitoring the neutrophil chemotaxis, superoxide anion release, and bactericidal activity.

Neonatal neutrophils demonstrated the proper qualitative FMLP-induced light scattering responses. This conclusion is supported by the following: (1) the shape and timing of the peaks of either the perpendicular rapid- or slow-LSR of neonatal cells were identical to those obtained with adult cells; (2) the light scattering responses of neonatal neutrophils yielded amplitude ratios of rapid- to slow-LSR and slow LSR to incident-LT tracings that highly corresponded to normal adult neutrophils.

Neonatal neutrophils did demonstrate quantitative abnormalities in their FMLP-induced LSR when compared with adult cells, however. The magnitude of neonatal neutrophil responses reached approximately only one-half that of corresponding adult LSR, irrespective of whether the response was assessed by the ''area under the peak'' parameter, or by direct recording of the amplitude of peaks (Table I). In addition, while adult cells displayed a relatively narrow variation in response amplitudes, neonatal responses demonstrated greater variability. The latter agrees with previous studies [10,22,39–41], and could be accounted for by the coexistence of different sub-populations in neutrophils of neonates, which might relate to various states of activation and/or maturation [29,40–42]. It is plausible to assume that the maturation of neonatal neutrophils during the postnatal period is quite variable.

Previous studies have shown that there is a monophasic increase in neonatal neutrophil LT, when stimulated by either FMLP or C5a, which is commonly interpreted as slow and irreversible cell aggregation [12,13,43]. In our study, the LT assay failed to distinguish between neonatal and adult neutrophils since we obtained similar increasing LT response patterns for both cell types when stimulated by FMLP at either 10 nM or 1 μM. In contrast, the two perpendicular scattering responses of the same cell suspensions were markedly decreased in neonatal cells. Within these responses, the rapid-LSR is correlated with membrane ruffling [44] and whole-cell polarization [29], while the slow-LSR is associated with the bactericidal activities of the neutrophils, and is physically accounted for by the overall increase in the cellular refractive index following the lysosomal secretion [23,45]. These results indicate that the interpretation of the LT response, which corresponds to the slow-LSR by dose response and kinetics, is less reliable than the perpendicular LSR, since it is composed of non-interacting, absorbed, and forward scattered-light. Furthermore, the correlation between increased LT and neutrophil aggregation has been already empirically invalidated [13,25]. We, therefore, conclude that the neutrophil inflammatory responsiveness is preferably monitored by the perpendicular LSRs.

The cellular and biochemical responses of the neutrophil to chemoattractant stimulation also revealed that the newborn neutrophils bear approximately only half the capacity of their counterpart adult cells. Reduced cell polarization and chemotaxis corresponded to the partial rapid-LSR, and the reduced lysosomal secretion and bactericidal activity ran parallel to the restrained slow-LSR [23]. However, superoxide anion production reached comparable levels in both newborn and adult cells, after stimulation by either the receptor-mediated FMLP, or the membrane-soluble PMA agent. This latter observation is sufficient to suggest that the FMLP signal perception at the receptor level of newborn infants is not different from that of adult cells.

Whole cell polarization of neonatal and adult neutrophils was similar when stimulated by low FMLP concentrations. Above 1 nM FMLP (optimal dose for chemotaxis stimulation), the neonatal cell polarization could not match the extent of the adult response. These observations suggest that neonatal neutrophils can respond to FMLP stimulation as much as adult cells, but their cytoskeletal framework fails to comply with the demands of a strong signal (i.e., higher than 10 nM FMLP). It should be noted that all inflammatory responses, which have been found to be impaired in the neonatal neutrophils, involve rearrangement of the cytoskeleton to one degree or another. Cytoskeleton rearrangement plays a direct role in cell polarization, chemotaxis, phagocytosis, and granule release (fusion with the plasma membrane after removal of the actin barrier). It should be stressed that the only function that appears to be fully active in neonatal neutrophils is cytoskeleton-free membrane associate-superoxide production.

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